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THE EFFECT OF WEAK BASES ON LYSOSOMAL ENZYME SECRETION BY MONONUCLEAR PHAGOCYTES

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Abstract—NH_4Cl induces a dose-dependent secretion of lysosomal enzymes by mouse peritoneal macrophages and human peripheral blood monocytes. The mechanism of NH_4Cl-stimulated hexosaminidase release is distinct from that initiated by the inflammatory stimulus, zymosan. The spontaneous lysosomal secretion of the continuous murine macrophage-like cell line, $P388D_1$, is inhibited by up to 50% in the presence of NH_4Cl and other weak bases.

The recognition of the macrophage as a predominant cell type in chronic inflammatory lesions [1] has led to a widespread acceptance of its importance in the pathogenesis of inflammation and to interest in the mechanisms by which this is effected. Considerable research has been directed toward identification of the range of substances which can be released by macrophages and their possible involvement in the initiation and maintenance of the inflammatory process. In particular, the correlation between the ability of certain stimuli, such as zymosan, to cause inflammation *in vivo* and to induce selective secretion of lysosomal hydrolases by macrophages *in vitro* has been used to suggest that these enzymes may have an important role in the tissue degradation which occurs in such pathological situations. More recently, it has been shown that other materials such as weak bases [2] also act as potent inducers of macrophage lysosomal enzyme release. However, while the range of secretory agents continues to extend, still little is understood of the mechanisms underlying lysosomal enzyme secretion by macrophages. It has been suggested that macrophages are capable of endogenous generation of C3b [3], a potent stimulator of lysosomal enzyme secretion when presented externally [4]. A correlation between the capacity of certain primary amines to initiate C3 breakdown and induce macrophage secretion has been taken as an indication of the possible role of C3b intracellularly in such enzyme release [2] although this was later discounted [5]. Interaction of certain stimulatory agents with macrophages induces activation of the hexose monophosphate shunt [6] but the direct stimulation of lysosomal enzyme release by this pathway has not been demonstrated. We have examined certain features of lysosomal hydrolase release by mononuclear phagocytes in response to zymosan and NH_4Cl using both mouse macrophages and human monocytes in culture.

In an earlier report [3] we demonstrated that the continuous murine macrophage-like cell line, $P388D_1$, secretes lysosomal enzymes spontaneously and is unresponsive to further stimulation by the usual inflammatory stimuli such as zymosan. In both respects this behaviour differs from the response of macrophages. In this paper we show that the effects of weak bases on $P388D_1$ secretion are also quite distinct from those on corresponding macrophage cultures, and attempt to interpret the data for both macrophage and $P388D_1$ cells in terms of current theories for the mechanism of lysosomal secretion in other cell types.

MATERIALS AND METHODS

Tissue culture materials. Plastic multi-well dishes (35 mm diameter) were from Costar (Cambridge, MA). Pig serum was obtained from Gibco Europe Ltd. (Paisley, U.K.). All other sera, tissue culture media and antibiotics were from Flow Laboratories (Irvine, U.K.). The sera were inactivated by heating at 56° for 30 min.

Biochemical reagents. Triton-X100, NH_4Cl, methylamine, dimethylamine and ethylamine were obtained from BDH (Poole, U.K.); zymosan from *Saccharomyces cerevisae*, chloroquine (diphosphate salt) and NADH were from Sigma Chemical Co. (Poole, U.K.); pyruvate (sodium salt) was from Boehringer Mannheim Gmbh (F.R.G.); *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside was obtained from Koch-Light Laboratories Ltd. (Colnbrook, U.K.); polystyrene latex spheres (0.81 μm diameter, 5% v/v) were from Difco (Detroit, MI).

Macrophage collection and culture. Macrophages were obtained by peritoneal lavage of normal Swiss mice (T.O. strain) as described previously [8]. The cells ($1.5–2.0 \times 10^6$/35 mm diameter tissue culture well) were incubated in medium 199 containing 10% (v/v) heat-inactivated pig serum, 100 I.U./ml penicillin and 100 μg/ml streptomycin at 37° and gassed with 5% CO_2 in air. After establishment of the cultures overnight, the medium was changed and the experimental treatments started as described in the text.

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Monocyte purification and culture. Human mononuclear cells were isolated from cell separator residues by the Ficoll-paque method [9] and monocytes further purified by selective adherence according to [10]. The cultures (approx 10^6 cells/35 mm diameter tissue culture well) were then maintained for 6 days in RPMI 1640 medium, 10% (v/v) heat-inactivated foetal calf serum, 100 I.U./ml penicillin and 100 μg/ml streptomycin at 37° and 5% CO_2 in air, with medium changes on days 4 and 6.

P388D$_1$ culture. The continuous murine cell line P388D$_1$ was maintained by bi-weekly passage in Eagle's minimum essential medium (EMEM) containing 10% (v/v) heat-inactivated foetal calf serum, 100 I.U./ml penicillin and 100 μg/ml streptomycin. For experimental cultures, 1.5×10^6 cells were seeded into 35 mm tissue culture dishes in 3 ml medium. The cells were allowed to adhere and spread for 1 hr before the medium was changed and the experimental treatment commenced.

Presentation of stimuli to cells. NH_4Cl, methylamine, dimethylamine, ethylamine and chloroquine were prepared as concentrated (100×) stock solutions in phosphate buffered saline and diluted into culture media. Zymosan was prepared as a 5 mg/ml stock solution in phosphate buffered saline and sonicated briefly before dilution into culture medium. Control media contained an equal volume of phosphate buffered saline. Latex particles [2 μl of the 5% (v/v) stock suspension/ml] were suspended directly in the culture medium. The medium pH was maintained at 7.2–7.4 by a bicarbonate/CO_2 buffer.

At the end of the incubation period of macrophage and monocyte cultures, medium was collected and the cells lysed in 1.5 ml phosphate buffered saline containing 0·1% (v/v) Triton-X 100 and removed by scraping with a silicone rubber bung. For P388D$_1$ cultures, the medium was removed and non-adherent cells sedimented in a bench centrifuge. Cells remaining attached to the dish were lysed in 0.1% (v/v) Triton-X 100, scraped off with a silicone rubber bung and combined with the pelleted cells.

Enzyme assays. β-*N*-Acetyl-D-glucosaminidase (hexosaminidase, EC 3.2.1.30) and lactate dehydrogenase (LDH, EC 1.1.1.27) were assayed as described in [8]. Results are expressed as means ± S.D. of triplicate cultures. Results are from single experiments and are representative of several separate experiments.

RESULTS

Induction of mononuclear phagocyte lysosomal enzyme secretion by NH_4Cl

Previous reports have suggested that mouse resident peritoneal macrophages selectively release several lysosomal hydrolases in response to *in vitro* incubation with various weak bases [2, 5]. The effect of NH_4Cl concentration on secretion of a lysosomal enzyme, hexosaminidase, by peritoneal macrophages during 5 hr incubation at 37° is shown in Fig. 1a. A dose-dependent release of hexosaminidase is measured at NH_4Cl concentrations greater than 10 mM. The selectivity of this process is shown by the absence of an equivalent simultaneous release of the cytosolic marker enzyme, lactate dehydrogenase. Since incubation of macrophage cultures with NH_4Cl concentrations in the range 0–50 mM does not alter the total hexosaminidase activity present (4407 ± 221 and 4634 ± 342 nmoles/hr/culture for 0 mM and 50 mM NH_4Cl incubations, respectively), it is inferred that NH_4Cl exerts its effect by altering the distribution of lysosomal enzymes rather than by any indirect effect via enzyme synthesis. These data agree with an earlier report [2] of NH_4Cl effects on β-glucuronidase and β-galactosidase secretion by peritoneal macrophages, although in this instance a larger proportion of the total activity was secreted. NH_4Cl incubation causes marked vacuolation of macrophages [11]. However, since similar expansion of lysosomal volume either by 80 mM sucrose [12] or latex spheres [13] does not induce secretion, it is unlikely that the effect of NH_4Cl on macrophage secretion is due to induced vacuolation alone.

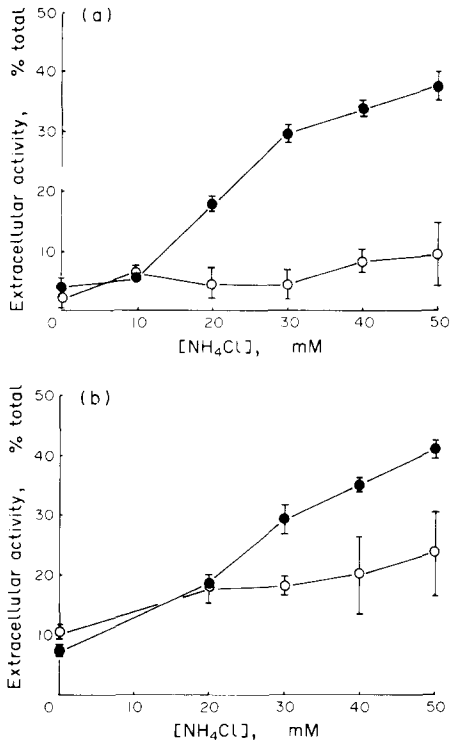

Fig. 1. Release of hexosaminidase during exposure of mononuclear phagocytes to NH_4Cl. (a) Mouse resident peritoneal macrophages were incubated for 5 hr in medium 199, 10% (v/v) heat-inactivated pig serum, 100 I.U./ml penicillin and 100 μg/ml streptomycin containing NH_4Cl at the concentrations indicated. (b) Human monocytes were incubated for 4 hr in RPMI 1640 medium, 10% (v/v) heat-inactivated foetal calf serum, 100 I.U./ml penicillin and 100 μg/ml streptomycin plus NH_4Cl at the concentrations shown. All cultures were incubated at 37° and gassed with 5% CO_2 in air. ●, hexosaminidase; ○, lactate dehydrogenase.

The sensitivity of human peripheral blood monocytes to NH_4Cl was also determined in a similar experiment (Fig. 1b). A selective dose-dependent secretion of hexosaminidase is induced in these cul-

tures also, although in this instance NH_4Cl is rather more lytic than in the corresponding macrophage cultures. Thus, although cultured monocytes are much less responsive than macrophages to the induction of lysosomal hydrolase secretion by particulate inflammatory agents such as zymosan and asbestos [14], these mononuclear phagocytes behave similarly when presented with NH_4Cl. This suggests that NH_4Cl may exert its effect on lysosomal secretion by a mechanism quite distinct from that of zymosan and other inflammatory mediators. The differences in the responses of monocytes (cf. macrophages) to zymosan stimulation [14] are not fully understood, but may reflect differences in the interactions of particles with the respective cell membranes.

Further evidence for separate sites of action of NH_4Cl and zymosan was obtained in experiments in which macrophages were stimulated maximally with zymosan and then exposed to NH_4Cl. Macrophages presented with zymosan rapidly internalise large numbers of these particles. If, following incubation with zymosan, extracellular particles are removed by washing, macrophages containing endocytosed particles continue to secrete lysosomal enzymes for several days [8]. Incubation of macrophages for 2 hr with 50 μg/ml zymosan produces the maximum rate of lysosomal secretion during a subsequent particle-free period, as judged by: (a) dose–response experiments (J. L. Bodmer, unpublished data); and (b) dependence between duration of exposure of macrophages to 50 μg/ml zymosan and the rate of secretion following removal of excess zymosan [8]. Table 1 shows the results of an experiment in which macrophages, pre-loaded with zymosan as described, were subsequently exposed to 50 mM NH_4Cl for 1 hr. In these cells, already responding maximally to zymosan stimulation, NH_4Cl promotes a further increment in the rate of hexosaminidase release independent of cell lysis (measured by LDH release). Further, the rate of secretion by these cells is numerically equal to the sum of the effects of zymosan and NH_4Cl exposures alone. In similar experiments, monocytes were exposed simultaneously to NH_4Cl and zymosan, the latter at a saturating concentration for stimulation of lysosomal enzyme secretion (Table 2). The data shown employs monocytes cultured in conditioned (mixed lymphocyte reaction [14]) medium, a treatment which we have previously shown to improve the recovery of cells during maturation without altering the nature of the secretory response to the above stimuli [14]. In common with macrophages, NH_4Cl and zymosan additively stimulated hexosaminidase secretion by monocytes.

Table 1. Effects of storage of zymosan on NH_4Cl-induced secretion of hexosaminidase by mouse peritoneal macrophages

	Enzyme release (as % of total activity)			
	Addition during phase I (0–2 hr)			
	None (medium only)		Zymosan (50 μg/ml)	
Addition during phase II (2–3 hr)	Hexosaminidase	LDH	Hexosaminidase	LDH
None (medium only)	12.0 ± 9.7	12.1 ± 3.1	36.9 ± 1.2	12.4 ± 0.4
NH_4Cl (50 mM)	42.9 ± 1.0	17.4 ± 3.4	74.6 ± 1.6	20.4 ± 4.7

Cultures were incubated for 2 hr in DMEM, 10% (v/v) heat-inactivated pig serum, 100 I.U./ml penicillin and 100 μg/ml streptomycin plus zymosan where appropriate. The cultures were then washed 4 times in phosphate buffered saline and re-incubated for 1 hr in fresh medium, containing NH_4Cl as indicated. The cultures were then harvested and assayed as described in Materials and Methods.

Table 2. Simultaneous stimulation of human monocyte lysosomal enzyme secretion by zymosan and NH_4Cl

	Extracellular activity (as % total)			
	Zymosan concentration			
	None (medium only)		50 μg/ml	
NH_4Cl concentration (mM)	Hexosaminidase	LDH	Hexosaminidase	LDH
0	23.4 ± 0.4	17.0 ± 2.1	33.6 ± 3.0	21.6 ± 2.3
30	35.2 ± 2.9	13.3 ± 1.5	75.5 ± 1.0	46.8 ± 3.7

Monocytes were cultured for 6 days in RPMI 1640 medium containing 10% (v/v) heat-inactivated foetal calf serum, 100 I.U./ml penicillin and 100 μg/ml streptomycin, plus 30% (v/v) conditioned medium for the last 2 days [14]. For measurement of secretion cultures were incubated for 4 hr in fresh medium containing NH_4Cl and zymosan as indicated. Media and cells were harvested and assayed as described in Materials and Methods.

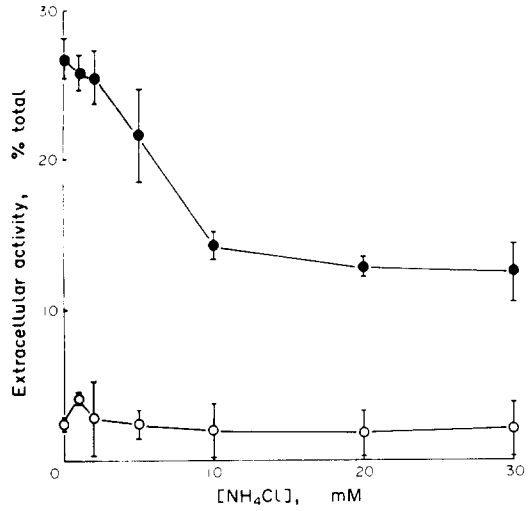

Fig. 2. Effects of NH_4Cl on hexosaminidase secretion by P388D_1 cells. Cultures were incubated for 24 hr in EMEM, 10% heat-inactivated foetal calf serum, 100 I.U./ml penicillin and 100 μg/ml streptomycin plus NH_4Cl at the concentrations shown. Total culture activities for 0 mM and 10 mM NH_4Cl cultures respectively were: hexosaminidase (nmole/culture/hr) 6030 ± 605, 6036 ± 258; lactate dehydrogenase (mU/culture) 830 ± 42, 830 ± 114. ●, Hexosaminidase; ○, lactate dehydrogenase.

Weak base effects on spontaneous lysosomal enzyme secretion by P388D_1 cultures

We have previously shown that the continuous macrophage-like cell line, P388D_1, secretes lysosomal hydrolases spontaneously and continuously in culture [7]. Further, inflammatory stimuli, such as zymosan and asbestos, which induce massive secretion by mouse macrophages [1] are unable to produce any increase in the rate of P388D_1 hexosaminidase release over that which occurs spontaneously [7]. In view of the preceding experiments, which suggest that zymosan and NH_4Cl may have different sites of action in macrophages, we were interested to determine the effect of NH_4Cl on lysosomal hydrolase secretion by P388D_1 cells. Figure 2 shows the response of P388D_1 hexosaminidase release during 24 hr incubation with several concentrations of NH_4Cl. These treatments did not affect cell viability as judged by LDH release. For the experiment shown, the control secretion rate in the absence of the agent is 26%/24 hr. Interestingly, and in contrast to its effects on macrophage and monocyte secretion, NH_4Cl inhibits P388D_1 hexosaminidase release in a dose-dependent manner. The maximum depression of secretion, to 14%/24 hr (i.e. approximately 50% of the control rate), is obtained at 10 mM NH_4Cl. For comparison, over the same incubation period 10 mM NH_4Cl stimulates macrophages to secrete 35% of their total hexosaminidase activity. The inhibition of lysosomal enzyme secretion of P388D_1 cells by NH_4Cl was not associated with any differences between the total culture activities of hexosaminidase in treated and control cells. This can most simply be interpreted by proposing that the effect of NH_4Cl is to produce a redistribution of activity between cells and extracellular medium, rather than to influence secretion by an effect on enzyme synthesis.

The effect of other weak bases on P388D_1 hexosaminidase secretion is shown in Table 3. Methylamine and ethylamine at 10 mM inhibit secretion to the same extent as NH_4Cl. Dimethylamine at this concentration and chloroquine at 50 μM produce severe vacuolation and extensive cell lysis, but at lower, non-lytic concentrations (1 mM and 10 μM respectively) also suppress lysosomal enzyme release. These data indicate that the effect of NH_4Cl on P388D_1 hexosaminidase secretion is most probably due to its properties as a weak base, rather than to any more specific function of the molecule. Table 4 shows the results of an experiment to determine whether P388D_1 secretion, when inhibited by NH_4Cl, can be re-elevated by zymosan stimulation. Latex spheres were included as an inert, particulate control. It was found that, like the normal spontaneous secretion of these cells, the rate of hexosaminidase release in NH_4Cl-treated P388D_1 cultures is not susceptible to stimulation by zymosan.

Table 3. Inhibition of P388D_1 lysosomal enzyme secretion by weak bases

Stimulus	Extracellular activity (% total)		Inhibition (%)
	Hexosaminidase	LDH	
Control (medium only)	26.3 ± 1.0	2.7 ± 1.8	—
NH_4Cl (10 mM)	14.0 ± 1.0	3.0 ± 2.9	47
Methylamine (10 mM)	13.4 ± 1.1	6.1 ± 1.9	49
Ethylamine (10 mM)	13.2 ± 0.8	5.1 ± 2.9	50
Dimethylamine (10 mM)	31.3 ± 3.2	17.4 ± 2.3	Lysis
(1 mM)	22.4 ± 1.1	4.8 ± 3.5	15
Chloroquine (50 μM)	30.7 ± 5.3	29.0 ± 5.6	Lysis
(10 μM)	18.9 ± 0.7	5.2 ± 0.9	28

Cultures were incubated for 24 hr in EMEM, 10% (v/v) heat-inactivated foetal calf serum, 100 I.U./ml penicillin and 100 μg/ml streptomycin containing weak bases at the concentrations indicated, then cells and media were harvested and assayed separately for hexosaminidase and lactate dehydrogenase. Inhibition of secretion is expressed as the rate of hexosaminidase release in the presence of each agent as a percentage of the control secretion rate.

Table 4. Effect of NH_4Cl on $P388D_1$ lysosomal enzyme secretion in the presence of particulate stimuli

Addition	Extracellular activity (% total)			
	Control		10 mM NH_4Cl	
	Hexosaminidase	LDH	Hexosaminidase	LDH
Control (medium only)	30.4 ± 1.9	5.6 ± 2.0	15.8 ± 2.9	5.4 ± 0.8
Zymosan (50 μg/ml)	35.5 ± 5.9	3.8 ± 0.7	18.1 ± 1.4	3.3 ± 2.4
Latex [2 μl/ml: 5% (v/v) stock]	30.5 ± 5.9	3.1 ± 0.2	18.9 ± 1.7	1.3 ± 1.1

$P388D_1$ cultures were exposed simultaneously to 10 mM NH_4Cl and/or various particles as indicated, in EMEM, 10% (v/v) heat-inactivated foetal calf serum, 100 I.U./ml penicillin and 100 μg/ml streptomycin. After 24 hr cells and media were assayed separately for hexosaminidase and lactate dehydrogenase.

DISCUSSION

This work shows that NH_4Cl induces a selective, concentration-dependent release of lysosomal hydrolase activity by mouse resident peritoneal macrophages and human peripheral blood monocytes. In view of earlier related observations of the ability of a range of weakly basic molecules (such as methylamine and chloroquine) to induce comparable lysosomal secretion by macrophages [2, 5], it is probable that the effect of NH_4Cl is also a consequence of its basicity, rather than any other more specific property of the molecule, such as its possession of an amine group. As far as we are aware, this is the first report of NH_4Cl-induced lysosomal hydrolase secretion by human monocytes, although we have already indicated [10] that chloroquine and ammonium acetate may stimulate monocyte hexosaminidase secretion.

Evidence that NH_4Cl may influence lysosomal secretion by a mechanism distinct from that of the inflammatory stimulus, zymosan, was obtained from two separate observations. Firstly, human monocytes cultured *in vitro*, which are relatively insensitive to stimulation of lysosomal enzyme release by zymosan, either when it is presented externally [14], or during intracellular storage of previously endocytosed particles [10, 14], remain capable of specific release of substantial amounts of hexosaminidase during exposure to NH_4Cl. In contrast to the effects of zymosan on monocytes, mouse macrophages storing endocytosed particles remain viable and continue to secrete lysosomal enzymes for prolonged periods [8]. Under conditions where the maximum rate of hydrolase secretion by storing macrophages is achieved, we have demonstrated that the cells remain susceptible to additional stimulation by NH_4Cl. This experiment provides the second argument for distinction between the sites of action of these two potent inducers of mononuclear phagocyte lysosomal enzyme release.

The biochemical events underlying NH_4Cl-induced secretion are not yet understood. It has been suggested that weak bases permeate into cells principally as neutral forms, and that within lysosomes these are trapped following protonation as the impermeable charged species [15]. As a consequence of the accumulation of weak bases within lysosomes, the intralysosomal pH rises [16] and water enters osmotically causing the organelles to swell to form large vacuoles [2, 11]. The secretory activity of NH_4Cl in macrophages is unlikely to be dependent solely on its ability to induce vacuolation, since other agents such as sucrose, concanavalin A and latex particles, all of which cause comparable expansion of lysosomal volume [11] but are not basic and do not significantly affect intralysosomal pH [16], fail to stimulate macrophage lysosomal hydrolase secretion [7, 12]. NH_4Cl and chloroquine are also effective in stimulating the secretion of lysosomal hydrolases by human fibroblasts [17–19]. For these cells it has been suggested that weak bases act indirectly on the postulated mannose-6-phosphate receptor-mediated system for routing of lysosomal enzymes from the Golgi into lysosomes, as a consequence of an elevation in lysosomal pH [20]. The overall effect is believed to be a diversion of newly-synthesised enzyme molecules into secretory vesicles rather than lysosomes, and thence to their release from the cell, because of a lack of unoccupied and available mannose-6-phosphate receptors on the lysosomal route. This may result from the intracellular mannose-6-phosphate receptors becoming saturated with bound enzyme, which at the elevated pH cannot dissociate [20]. It is less easy to envisage how such a system could explain the mechanism of mononuclear phagocyte NH_4Cl-induced lysosomal secretion. Even if the existence of a similar packaging system is demonstrated, the apparent independence of weak-base stimulated macrophage secretion from *de novo* enzyme synthesis (W. Jessup, unpublished observations); [1]) requires substantial modification of the above model for fibroblast enzyme release.

$P388D_1$ is a continuous murine tumour cell line which possesses several properties normally considered characteristic of mononuclear phagocytes. It is therefore increasingly used as a convenient model system for the study of macrophage metabolism. However, this work and earlier observations [7] suggest that there exist significant qualitative differences between the behaviour of $P388D_1$ cells and primary macrophage cultures, at least in the regulation of their lysosomal enzyme secretion. For example, unlike macrophages, $P388D_1$ cultures secrete lysosomal hydrolases spontaneously and continuously. In this respect, $P388D_1$ secretion shows a superficial resemblance to that of macrophages storing previously endocytosed inflammatory stimuli [8]. However, while the latter are additionally stimulated by exposure to NH_4Cl, $P388D_1$ secretion is depressed by incubation with the same agent.

The mechanism of spontaneous lysosomal enzyme

release by P388D$_1$ cells is not understood. Secretion may be due to the absence or dysfunction of a ligand on newly-synthesised enzyme molecules, leading to a failure of the cells to segregate lysosomal materials from secretory products, such as occurs in I-cell disease [20]. Alternatively, the complementary receptor may be reduced in amount, or availability, or have a reduced affinity for, its ligand. Another possibility is that P388D$_1$ cells package their lysosomal enzymes normally but, unlike macrophages, have a self-activated triggering mechanism for release.

The depression of the constitutive lysosomal secretion observed when P388D$_1$ cells are exposed to weak bases may be the result of a general effect on membrane cycling. NH_4Cl significantly reduced fluid endocytosis in rat yolk sacs [21] and protein secretion by hepatocytes [22]. An equivalent effect on exocytosis in P388D$_1$ cultures might reduce the rate of hexosaminidase secretion. On the other hand, weak bases may act selectively on receptor-mediated systems, as has been proposed for the other cell types [20]. In any cell type in which weak bases induce an increase in the proportion of intracellular mannose-6-phosphate receptors which are occupied, a consequence of this increase will probably be an increase in the amount of lysosomal enzyme so bound. This will presumably require that an increased proportion of newly-synthesised enzyme binds to these receptors. During the period of increasing occupancy, less new enzyme would be available for secretion. In P388D$_1$ cells this period might be that described in this paper in which NH_4Cl depresses secretion; in fibroblasts this phase may be so short as to have escaped attention.

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Note—Since this work was completed, we have shown that P388D$_1$ spontaneously secreted hexosaminidase is in the 'high-uptake' form [23] and phosphorylation of P388D$_1$ β-glucuronidase has been demonstrated directly by another group [24]. This suggests that P388D$_1$ lysosomal enzyme secretion is more likely to be due to a dysfunction in the complementary receptor in these cells.